

Royalactin extends lifespan of *C. elegans* through epidermal growth factor signaling

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Abstract

Royalactin is a glycoprotein essential for the development of long-lived queen honeybees. Only larvae fed with royal jelly, containing royalactin, develop into queens. Royalactin plays a central role in this process by switching on the epidermal growth factor (EGF) receptor signaling pathway which ultimately leads to epigenetic changes and a long-lived queen phenotype. Recently it was shown that royalactin by itself also extends lifespan in *Drosophila melanogaster*. Yet, the mechanism by which royalactin promotes longevity remains largely unknown.

We set out to characterize the effects of royalactin on *Caenorhabditis elegans* lifespan, and clarify the possible involvement of EGF signaling in this process. We demonstrate that royalactin extends lifespan of this nematode and that both EGF (LIN-3) and its receptor (LET-23) are essential to this process. To our knowledge, this is the first report of royalactin-mediated lifespan extension in a non-insect species. Additionally, we show that royalactin enhances locomotion in adult nematodes, implying that royalactin also influences healthspan.

Our results suggest that royalactin is an important lifespan-extending factor in royal jelly and acts by promoting EGF signaling in *C. elegans*. Further work will now be needed to clarify which (secondary) signaling pathways are activated by royalactin, and how this ultimately translates into an extended health- and lifespan.

Disclaimer

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Keywords

Royal jelly, Dietary intervention, EGF signaling, Aging, Invertebrate, Healthspan

Abbreviations

| | |
|-------------|---|
| 10-HDA | 10-hydroxy-2-decenoic acid |
| BSA | Bovine Serum Albumin |
| dRoyalactin | Deglycosylated royalactin |
| dsRNA | Double-stranded RNA |
| EGFR | Epidermal growth factor receptor |
| hRoyalactin | Heat-treated royalactin |
| MRJP1 | Major Royal Jelly Protein 1 (royalactin) |
| NGM | Nematode growth medium |
| pBSA | Thermolysin-treated BSA |
| pRoyalactin | Thermolysin-treated royalactin |
| RJ | Royal jelly |
| RNAi | RNA interference |
| RP-HPLC | Reversed-phase high-performance liquid chromatography |
| SEM | Standard error of the mean |

1. Introduction

Aging, the gradual loss-of-function over time which leads to increased sensitivity to intrinsic and external stressors and ultimately death, is the main risk factor for many prominent and debilitating diseases in modern society including cancer and dementia. By modulating the aging process itself, it might be possible to delay or prevent these diseases (Butler et al. 2008; Le Couteur et al. 2012). Since the discovery that aging can be altered by modulating certain conserved genetic pathways and biochemical processes (Klass 1983; Kenyon 2010; López-Otín et al. 2013), scientists have been looking for promising pharmaceutical compounds that act on these pathways to confer increased longevity. Such a compound, if effective, could bring more health benefits than any dietary manipulation or lifestyle studied to date (de Magalhães et al. 2012).

In search for a compound that might delay aging, one can rely on information from naturally long-lived organisms. The western honeybee *Apis mellifera* displays a strong polyphenism with regard to aging: the fertile queen caste is long-lived and typically reaches 1-2 years whereas the infertile worker caste only lives 3-6 weeks (Page and Peng 2001). While genotypic effects can play a minor role (Lattorff and Moritz 2013), the main factor that determines whether a honeybee larva develops into a queen or worker is its nourishment. Only those larvae that are continuously fed with royal jelly (RJ) develop into queens (Haydak 1970). As RJ is the sole food source for queens throughout their lifetime, the question arises whether RJ contains the compound(s) responsible for the queen's long-lived phenotype.

Whereas RJ is a complex mixture of sugars, proteins, lipids and vitamins (Takenaka 1982), it was recently shown that the RJ protein royalactin is essential for queen differentiation (Kamakura 2011). Royalactin activates the epidermal growth factor receptor (EGFR) signaling pathway in honeybee larvae, which ultimately leads to epigenetic changes and queen development. Moreover, Kamakura (2011) showed that royalactin shortens development time and increases longevity in the fruit fly *Drosophila melanogaster* through the EGFR signaling pathway, thus demonstrating that the glycoprotein also confers its beneficial effects on another insect species.

RJ has previously been shown to extend lifespan in mice (Inoue 2003) and in the nematode *Caenorhabditis elegans* (Honda et al. 2011). However, the primary factor responsible for this lifespan extension remained elusive. Interestingly, one of the main conclusions drawn from the study on *C. elegans* was that RJ proteins are not likely contributors to the lifespan-extending effect of RJ (Honda et al. 2011), implying that royalactin may not be able to extend lifespan in *C. elegans*.

We employed the model organism *C. elegans* to further study the effect of royalactin on longevity. Contrary to the previous report that RJ-mediated longevity is likely not due to RJ proteins (Honda et al. 2011), we show that royalactin is able to extend nematode lifespan in absence of other RJ constituents. To our knowledge, this is the first report of royalactin-mediated lifespan extension in a non-insect species. Additionally, we show that royalactin increases adult swimming activity in *C. elegans* and we partly elucidate the mechanism through which it exhibits its lifespan-extending effect.

2. Materials and Methods

2.1 Royalactin extraction

RJ, produced by worker honeybees (*Apis mellifera*, hybrid variety 'Buckfast'), was purchased in a frozen state from the beekeeper Peter Otte (Graide, Belgium). Royalactin was extracted from RJ by multiple rounds of ultracentrifugation, as described by Simuth (2001), followed by reversed-phase high-performance liquid chromatography (RP-HPLC). The different RP-HPLC fractions were lyophilized and analyzed by peptide mass fingerprinting using an Ultraflex II matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (Bruker Daltonics). All fractions uniquely identified as MRJP1 were pooled and stored at -20°C before usage in the lifespan experiments (for more details on the methods used see appendix A.1 - A.6). We confirmed that these pooled fractions predominantly contain the monomeric form of MRJP1, also known as royalactin (appendix A.7).

2.2 Thermolysin treatment of royalactin

Part of the purified royalactin was treated with the metalloprotease thermolysin (EC 3.4.24.27, Sigma, T7902). This reaction was performed in thermolysin digestion buffer (50 mM Tris, 10 mM CaCl₂, 1M NaCl, pH 8.0) at an enzyme-to-substrate ratio of 1:20 during 4 h 15 min, while shaking at 70°C. Bovine Serum Albumin (BSA, Sigma, A7906) was treated likewise in a parallel reaction. After freeze-drying, the efficacy of these treatments was verified via mass spectrometric analysis (appendix A.5).

2.3 Deglycosylation of royalactin

We removed the N-linked oligosaccharides from royalactin with the glycosidase PNGase F (Sigma, P7367). The reaction was performed under non-denaturing conditions in sodium phosphate buffer (100 mM, pH 7.0) with 30 U of PNGase F mg⁻¹ protein, during 60 h at 37°C. Amicon Ultra-0.5mL 30K centrifugal filters (Millipore) were used to separate the

oligosaccharides from deglycosylated royalactin (dRoyalactin). We verified successful deglycosylation via SDS-PAGE (appendix A.8). To take into account potential heat degradation of royalactin, we subjected royalactin to identical incubation and processing steps in the absence of PNGase F. The resulting product is referred to as heat-treated royalactin (hRoyalactin).

2.4 *C. elegans* maintenance

The ZB2844 *hpa-1(tm3256)* and N2 *wild-type Bristol* strains were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, USA). Worms were cultivated at 20°C on standard nematode growth medium (NGM) plates with *Escherichia coli* OP50 as a food source.

2.5 Lifespan experiments

We tested the effects of royalactin, thermolysin-treated royalactin (pRoyalactin) and deglycosylated royalactin (dRoyalactin) on the lifespan of *C. elegans*. All lifespan experiments were performed at 20°C on regular NGM plates or on NGM plates supplemented with royalactin and/or BSA (both intact or treated with thermolysin and/or heat; see appendix B.1).

2.5.1 Preparation of NGM agar containing royalactin

To evaluate the optimal dosage, intact royalactin was added to liquid NGM at three different final concentrations: 0.04, 0.4 and 1.6 $\mu\text{g mL}^{-1}$. pRoyalactin was similarly added to NGM at two distinct final concentrations: 0.4 and 1.6 $\mu\text{g mL}^{-1}$. To exclude nutritional effects, BSA (or thermolysin-treated BSA: pBSA) was added to every condition such that the final total concentration of protein was 5 $\mu\text{g mL}^{-1}$ (Table B.1). Two controls were used in which solely 5 $\mu\text{g BSA mL}^{-1}$ or no additional protein at all was added to the medium. In the latter case, an equal volume of Milli-Q water was added. All plates in the initial experiment were seeded with *E. coli* OP50 as a food source.

Subsequent lifespan experiments used a concentration of either 1.5 or 1.6 $\mu\text{g royalactin mL}^{-1}$ (Tables B.2, B.3 and B.4) and plates were seeded with *E. coli* HT115(DE3) containing the appropriate plasmid construct (see 2.5.4).

2.5.2 Age synchronization

Worms were synchronized by isolating eggs from gravid adults through hypochlorite treatment (Wood 1988), followed by a sucrose density centrifugation step to separate the eggs from cellular debris. Eggs were washed at least four times with S-basal buffer (0.1 M

NaCl, 44 mM KH₂PO₄, 6 mM K₂HPO₄) and incubated overnight at 20°C while rotating in S-basal.

2.5.3 Determination of lifespan

Synchronous L1 animals were kept on 9.5 cm-diameter NGM plates until they reached the young adult stage. Eighteen to twenty-five individuals were then transferred to each assay plate (3.5 cm-diameter), corresponding to a total of 72 to 100 individuals per condition. During the initial lifespan experiment, animals were transferred to fresh assay plates every day until the end of their reproductive period and every 3 days thereafter. In the other experiments, in which 5-fluoro-2'-deoxyuridine (Sigma) was used to bypass the high rates of internal hatching inherent to the carried out RNAi knockdowns (appendix B.4), animals were transferred once every 3-4 days.

Lifespan was monitored daily starting from the first day of the adult stage (scored as day 0). Animals that did not move when gently prodded with a platinum wire were marked as dead. Animals that crawled off the plate or died of vulval bursting or internal hatching were censored.

2.5.4 RNAi treatment of worms

Via regular feeding RNAi (Rual et al. 2004) we knocked down *lin-3* (*C. elegans* EGF-encoding gene) and *let-23* (*C. elegans* EGFR-encoding gene). *E. coli* HT115(DE3) producing dsRNA for the individual target genes were seeded onto NGM plates containing 1 mM isopropyl β-D-1-thiogalactopyranoside and 50 µg mL⁻¹ ampicillin. As a negative control we seeded plates with HT115(DE3) carrying the empty pL4440 vector. These bacteria produce dsRNA corresponding to the multiple cloning site of the pL4440 vector, which has no known target in *C. elegans* (Rual et al. 2004). As a positive control for the RNAi setup we used HT115(DE3) transformed with pL4440(*unc-22*), which leads to an easily recognizable twitching phenotype upon successful RNAi induction in *C. elegans* (Fire et al. 1998). All RNAi constructs were sequenced to verify the identity of the insert (appendix B.3).

An in-house RNAi construct, referred to as pL4440(*let-23*), was generated against *let-23* since the only available RNAi clone was incorrectly annotated in the Ahringer RNAi feeding library (Qu et al. 2011; appendix B.3). The *lin-3* and *unc-22* RNAi clones were derived from the *C. elegans* ORF-RNAi library v1.1 (Rual et al. 2004). Worms were raised on RNAi plates from the L1 stage onwards throughout development. Lifespan assays were performed as described in 2.5.3, providing worms with fresh RNAi assay plates every 3-4 days.

2.5.5 Statistical analysis

We used *R* (R Development Core Team 2013) to construct survival curves (Kaplan-Meier), carry out all related statistical analyses and calculate the mean lifespan and standard error of the mean (SEM). Kaplan-Meier survival curves were statistically analyzed using a Cox Proportional Hazard (to compare multiple conditions) or a log-rank test (to compare a single curve to control). The corresponding *p*-values are referred to as p_{cox} and $p_{\text{log-rank}}$.

2.6 Locomotion measurements

We measured swimming activity of *C. elegans*, using an automated infrared tracking system, as described by Meelkop et al. (2012) with a few modifications. In short: adult nematodes (day 3.5 of the adult stage) were added to wells of a flat-bottom 96-well plate containing 200 μL of S-complete medium (Solis and Petrascheck 2011) with a final concentration of 85 μM 5-fluoro-2'-deoxyuridine to prevent self-reproduction, and 5 $\mu\text{L mL}^{-1}$ nystatin (Sigma, N1638) and 50 mg mL^{-1} ampicillin to avoid contamination. As a food source we added flash frozen *E. coli* K12 bacteria (Artechno, Isnes, Belgium) until an OD_{600} of 1.7 was reached. For the treated condition, worms were grown on royalactin-containing NGM plates starting from the L1 stage (analogous to the lifespan experiments) until transferring them to liquid medium with a final concentration of 1.7 $\mu\text{g mL}^{-1}$ royalactin. Fourteen wells were used for each condition, with ca. 30 worms in each well. After a recovery period of three hours, we measured locomotion during 24 h with the WormMicrotracker (Designplus) (Simonetta and Golombek 2007). Activity bins were added up for each well. The results were analyzed using a one-way ANOVA followed by a Tukey's HSD *post hoc* test.

3. Results

3.1 Royalactin influences *C. elegans* lifespan

3.1.1 No significant effect of BSA on *C. elegans* lifespan

To exclude that lifespan extension due to royalactin supplementation would be merely the result of a higher amount of protein in the growth medium, we followed the life course of worms exposed to BSA. We were unable to detect significant differences in survival between worms treated with 5 $\mu\text{g BSA mL}^{-1}$ and the control (Fig. 1.A; Table 1), indicating that under the experimental conditions used, protein supplementation by itself has no significant influence on *C. elegans* lifespan.

3.1.2 Royalactin extends *C. elegans* lifespan

Royalactin extends lifespan of *C. elegans* at all three concentrations tested (Fig. 1.A), compared to both controls in which either no protein or BSA was added to the medium. With increasing concentration of royalactin, the mean lifespan of *C. elegans* rose with 26.8%, 29.1% and 33.9%, respectively (Table 1). Though there was no significant difference in survival between these three conditions, we observed the strongest lifespan-extending effect at a concentration of 1.6 μg royalactin mL^{-1} (Fig. 1.A).

3.1.3 pRoyalactin does not extend *C. elegans* lifespan

Adding thermolysin-treated royalactin (pRoyalactin) to the medium has no significant influence on *C. elegans* lifespan (Table 1; Fig. 1.B), compared to adding thermolysin-treated BSA (pBSA). There is a slight, albeit non-significant, increase in mean lifespan that is clearly not as pronounced as with intact royalactin. Thus, the strong lifespan-extending effect of royalactin is abolished after thermolysin-treatment.

3.1.4 Both dRoyalactin and hRoyalactin extend *C. elegans* lifespan

To assess whether the N-linked oligosaccharides from royalactin are needed for its lifespan-extending effect, we removed them from royalactin (see 2.3). Deglycosylated royalactin (dRoyalactin) and mildly heat-treated royalactin (hRoyalactin) are both still capable of extending *C. elegans* lifespan (Fig. 1.C; Table 1) to the same extent as intact royalactin. Supplementation of NGM with oligosaccharides, which were removed from royalactin, does not result in an increase in longevity, compared to the control (Fig. 1.C; Table 1). When comparing the oligosaccharide and dRoyalactin condition, we do see a significant difference in survival (+19% mean lifespan; $p_{\text{log-rank}} = 3.26\text{E-}4$). Taken together, these results indicate that the N-linked carbohydrate moieties on royalactin are not needed for its lifespan-extending effect.

3.2 Knockdown of the EGF receptor or its ligand abolishes the lifespan-extending effect of royalactin

Based on its known dependence on EGFR signaling in insects (Kamakura 2011), we tested the ability of royalactin to influence *C. elegans* lifespan after knockdown of *lin-3* (EGF) or *let-23* (EGFR).

As a negative control for the RNAi experiments we used *E. coli* HT115(DE3) bacteria, carrying the empty pL4440 vector, as a food source for wild-type *C. elegans* (see 2.5.4). As expected from the initial lifespan assay (see 3.1), worms treated with 1.5 μg royalactin mL^{-1} NGM live significantly longer than those treated with BSA only (Fig. 2.A and Fig. B.1). Mean lifespan is increased by 17.5% (Table 2). A similar increase in longevity is seen for worms treated with 1.6 μg royalactin mL^{-1} (Fig. 2.B and Fig B.6; Table 2). Thus, the lifespan-

extending effect of royalactin is maintained when culturing *C. elegans* in the presence of *E. coli* HT115(DE3) expressing control dsRNA.

The genes *lin-3* and *let-23* encode the EGF ligand and EGFR, respectively. By itself, knockdown of *lin-3* causes no significant difference in survival (Fig. B.3). However, *lin-3* knockdown clearly abolishes the lifespan-extending effect of royalactin (Fig. 2.A; Table 2). In other words: when knocking down *lin-3*, administering royalactin to *C. elegans* no longer increases mean or median lifespan. Similarly, knockdown of *let-23* – which drastically decreases survival under standard conditions (-26.8% mean lifespan; Fig 2.B; Fig. B.5) – abolishes the lifespan-extending effect of royalactin. During *let-23* RNAi, royalactin treatment no longer increases mean or median lifespan of *C. elegans* (Fig. 2.B; Table 2).

Our data thus demonstrate that the epidermal growth factor (LIN-3) and its receptor (LET-23) are essential for royalactin-mediated lifespan extension in *C. elegans*.

3.3 Royalactin enhances locomotion in *C. elegans*

We compared swimming activity between royalactin-treated and non-treated wild-type worms in early to mid-adulthood (from day 3.5 until 4.5 adult) using an automated tracking system. As a positive control we used the *hpa-1(tm3256)* deletion mutant (*high performance in advanced age*) which displays an increased amount of body bends in liquid medium (Iwasa et al. 2010).

Both royalactin-treated and positive control worms show an increase in swimming activity compared to the wild-type control ($p = 5.41\text{E-}9$ and $1.50\text{E-}5$, respectively). Average swimming activity increases with ca. 75% upon royalactin treatment (Fig. 3). Additionally, royalactin-treated worms display a significant increase in average swimming activity compared to the positive control (+20%; $p = 5.46\text{E-}3$).

4. Discussion

Due to the success of modern medicine, many human populations enjoy a substantially increased life expectancy. These ‘added years’ are nevertheless to be balanced with the risk of developing many diseases, which increases exponentially with age. If we are to care for our elderly, we need to invest in preventing or at least delaying these detrimental age-related physiological changes. One way to approach this, is by modulating the fundamental mechanisms of aging (Kirkland 2013). This can be achieved by finding compounds that

target the aging process across different phyla (Fontana et al. 2010; de Magalhães et al. 2012; Lucanic et al. 2013).

4.1 Royalactin confers lifespan-extending properties to royal jelly

Royalactin, also known as the monomeric form of major royal jelly protein 1 (MRJP1), apalbumin-1 and RJP-1, is a glycoprotein unique to *A. mellifera* and the most abundant protein in RJ (Schmitzová et al. 1998; Buttstedt et al. 2014). In 2011, Honda et al. revealed that RJ extends lifespan in the nematode *C. elegans*. We demonstrate that royalactin by itself is capable of extending lifespan in *C. elegans*. To our knowledge, this is the first report of royalactin-mediated lifespan extension outside of the insect order. While the highest concentration of royalactin provoked the biggest lifespan-extending effect, no clear dose-dependency can be concluded from this study. Supplementation with the protein BSA induces no response, which argues against the possibility that the effects of royalactin are simply nutritional. Treating royalactin simultaneously with thermolysin and heat (i.e., disrupting the native protein structure) abolishes its lifespan-extending effect, further supporting this notion.

While N-linked glycosylation can play a major role in protein oligomerization and stability (Elbein 1991), this modification does not appear to be essential for royalactin to promote longevity. Our results show that dRoyalactin extends lifespan in *C. elegans*, as strongly as hRoyalactin, while the isolated carbohydrate moieties have no such effect. This is line with previous reports showing that deglycosylated royalactin retains its stimulatory effect on rat hepatocytes (Kamakura 2002) and that recombinant royalactin (without carbohydrate moieties) still induces queen differentiation in *A. mellifera*, has stimulatory effects on murine macrophages (Majtán et al. 2006) and increases lifespan in *D. melanogaster* (Kamakura 2011).

In literature, part of the lifespan-extending effect of RJ has been attributed to 10-hydroxy-2-decenoic acid (10-HDA), a fatty acid (Honda et al. 2011). Our results suggest that royalactin, rather than 10-HDA, is the main lifespan-extending factor in RJ. Royalactin increases the mean lifespan of wild-type *C. elegans* by 18-34% (depending on the concentration, the bacterial food source and agar composition), while the maximum increase in mean lifespan with 10-HDA remains limited to 12% (Honda et al. 2011). Since only part of the lifespan-extending effects of RJ can be attributed to 10-HDA, there are in all likelihood other longevity-promoting compounds contained in RJ. While Honda et al. (2011) argued that these compounds are unlikely to be intact proteins, we here contrastingly show that RJ-derived royalactin can extend longevity in *C. elegans*. However, Honda et al. (2011) do suggest that specific proteinaceous fragments (such as peptides and/or proteolysis

byproducts) may be in part responsible for the lifespan-extending properties of RJ. This may imply that it is not full royalactin, but a specific peptide derived from royalactin that promotes longevity. Our attempts to generate such a fragment using the protease thermolysin were unsuccessful thus far. A highly specific combination of proteases and/or incubation times may be needed to generate said fragment.

It is conceivable that 10-HDA plays an auxiliary role in lifespan extension by RJ, for example through its histone deacetylase inhibitor activity (Spannhoff et al. 2011; Buttstedt et al. 2014). It has been shown that epigenetic alterations (e.g. histone modifications) can play an important role in the regulation of the aging process of *C. elegans* and other organisms (de Magalhães et al. 2012; López-Otín et al. 2013). It would be interesting to investigate whether royalactin and 10-HDA have additive effects on modulating lifespan, and if these substances induce longevity-associated epigenetic changes.

4.2 Mode of action of royalactin: the epidermal growth factor receptor signaling pathway

Royalactin activates the EGFR signaling pathway in *D. melanogaster*, *A. mellifera* and primary rat hepatocytes (Kamakura 2002; Kamakura 2011). This pathway has in recent years been identified as a crucial regulator of the aging process in *C. elegans* (Yu and Driscoll 2011; Rongo 2011). Moreover, the lifespan increase evoked by royalactin is very similar to the one seen in the EGF receptor gain-of-function mutant *let-23(sa62)* (Liu et al. 2011).

These observations led us to investigate the role of EGF signaling in royalactin-mediated lifespan extension. Here, we show that knockdown of either *lin-3* (EGF) or *let-23* (EGFR) completely abolishes royalactin-mediated lifespan extension. This is the first experimental indication that royalactin acts through the EGFR signaling pathway in *C. elegans*.

Despite the efforts of Kamakura (2011) to characterize the interplay between royalactin and the EGFR in *D. melanogaster*, it remained unclear how royalactin interacts with this receptor. It is unknown whether royalactin by itself is a ligand of the EGFR, or whether it somehow helps endogenous ligand(s) to activate the EGFR. Since we showed that EGF (LIN-3) is essential for royalactin-mediated lifespan extension in *C. elegans*, this points to a mechanism wherein royalactin by itself is no ligand of the EGFR, but somehow interacts with LIN-3 to promote EGF signaling. One plausible explanation is that royalactin (or a fragment thereof) binds to LIN-3, which in turn promotes the binding of the ligand to the extracellular domain of the EGFR and thus enhances EGF signaling. Another possibility is that royalactin acts on

regulators of EGF signaling (such as HPA-1) and/or promotes the release of LIN-3, which is synthesized as a membrane-anchored precursor.

Although EGF signaling is a known regulator of aging in *C. elegans*, exactly how royalactin treatment results in increased longevity remains to be elucidated. It is unknown which (secondary) signaling pathways are activated and how this translates into an extended lifespan. One possibility is that royalactin ultimately helps to maintain protein homeostasis (proteostasis), known to support healthy aging. Enhanced EGF signaling is directly linked to sustaining proteostasis in adult *C. elegans* (Liu et al. 2011; Rajalingam and Dikic 2011; Rongo 2011) while impaired proteostasis is one of the hallmarks of aging (López-Otín et al. 2013).

While royalactin is unique to *A. mellifera*, its target, the EGFR pathway, is highly conserved in evolution from *C. elegans* to humans (Lemmon and Schlessinger 2010). This implies royalactin might have the potential to exert its effects in a wide range of organisms, including mammals. Previous studies already showed immunostimulatory effects of royalactin (and oligomeric MRJP1) on mice macrophages and human monocytes (Kimura et al. 1995; Majtán et al. 2006), as well as enhanced proliferation of rat liver cells via increased EGFR signaling (Kamakura 2002). However, a note of caution is due: while a small increase in EGFR activity could prove beneficial for healthy aging, augmented EGFR signaling is also associated with epithelial cancers (Rajalingam and Dikic 2011). Further research will have to clarify whether the beneficial effects of royalactin on longevity or other traits might also be present in other organisms.

4.3 Royalactin and healthspan

Since the link between royalactin and lifespan extension has only recently been established, there are still many unanswered questions. Little is known about whether royalactin also positively impacts other traits of living organisms and genuinely results in healthy aging, rather than simply prolonging an aged state.

Royalactin increases fecundity in *D. melanogaster* (Kamakura 2011), so it would be interesting to investigate whether royalactin has a similar influence on *C. elegans*. The EGFR - which is stimulated by royalactin - regulates reproduction and fertility in adult worker honeybees (Formesyn et al. 2014). This shows that EGF signaling is not only needed during larval development, but also plays an important role in regulating fertility during adulthood - at least in the honeybee.

Since royalactin seems to activate the EGFR in *C. elegans*, it is likely that royalactin not only increases lifespan but also healthspan of *C. elegans*. The activation of LET-23 is associated

with maintaining muscle function and integrity, a reduced accumulation of age pigments and a higher rate of locomotion during old age (Iwasa et al. 2010; Yu and Driscoll 2011). Similarly, we showed that royalactin-treatment positively influences the rate of locomotion during early to mid-adulthood (see 3.3), implying that royalactin also extends nematode healthspan. Our observations point to a compression of morbidity until near the end of life, rather than merely an extended lifespan in poor health.

4.4 Promising developments and perspectives

It seems conceivable that in the future, clinical interventions might be developed that could delay or prevent age-related changes in humans (de Magalhães et al. 2012; Kirkland 2013). Some of these interventions could be based on natural products (like RJ), extracts thereof (such as royalactin or 10-HDA) or other compounds that target key regulators of the aging process.

Phase III clinical trials are already underway to assess the effects of RJ on elderly people (Niu et al. 2013). Preliminary results show that the daily intake of RJ improves muscle strength and physical performance in elderly volunteers, as seen in mice both *in vitro* and *in vivo* (Niu et al. 2013). Yet, care should be taken since sufficient data are lacking. Furthermore, since RJ can contain a number of contaminants, such as antibiotics, that may cause possible adverse reactions (Bogdanov 2005), it might not be suited as an efficient method of treatment in its current form. In this context, it is paramount to identify the actual component(s) of RJ that result in an extended lifespan and healthspan. Our results indicate that royalactin is at least one of these compounds. Moreover, we show that the N-linked oligosaccharides of royalactin – which are putative human allergens in RJ (Hayashi et al. 2011) – are not required to promote longevity, which brings it one step closer to being used as a nutraceutical.

Additionally, analysis of the involved molecular mechanisms could identify new drug targets for longevity promotion (de Magalhães et al. 2012). As such, we believe a better understanding of the interaction of royalactin with genetic pathways and biochemical processes conserved in evolution might contribute to the long-term development of therapeutic interventions that target the human aging process. Modulating the fundamental mechanisms of aging – to delay several age-related diseases as a group, rather than one at a time – is a promising approach that is quickly gaining traction (Kirkland 2013).

5. Conclusions

Taken together, our results suggest that the glycoprotein royalactin is an important lifespan-extending factor in royal jelly and acts by promoting EGF signaling. Both EGF and its receptor seem to be essential for royalactin to exert its positive effects on longevity, whereas the N-linked oligosaccharides of royalactin are not required for its activity. Further work will now be needed to clarify which (secondary) signaling pathways are activated by royalactin, and how this ultimately translates into an extended health- and lifespan.

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References

- Bogdanov S (2005) Contaminants of bee products. *Apidologie* 37:1–18. doi: 10.1051/apido:2005043
- Butler RN, Miller RA, Perry D, et al. (2008) New model of health promotion and disease prevention for the 21st century. *BMJ* 337:a399. doi: 10.1136/bmj.a399
- Buttstedt A, Moritz RFA, Erler S (2014) Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews* 89:255–269. doi: 10.1111/brv.12052
- Le Couteur DG, McLachlan AJ, Quinn RJ, et al. (2012) Aging biology and novel targets for drug discovery. *The journals of gerontology Series A, Biological sciences and medical sciences* 67:168–174. doi: 10.1093/gerona/glr095
- Elbein AD (1991) The role of N-linked oligosaccharides in glycoprotein function. *Trends in biotechnology* 9:346–352.
- Fire A, Xu S, Montgomery MK, et al. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–811. doi: 10.1038/35888
- Fontana L, Partridge L, Longo VD (2010) Extending healthy life span--from yeast to humans. *Science* 328:321–326. doi: 10.1126/science.1172539
- Formesyn EM, Cardoen D, Ernst UR, et al. (2014) Reproduction of honeybee workers is regulated by epidermal growth factor receptor signaling. *General and comparative endocrinology* 197:1–4. doi: 10.1016/j.ygcen.2013.12.001
- Hayashi T, Takamatsu N, Nakashima T, Arita T (2011) Immunological Characterization of Honey Proteins and Identification of MRJP 1 as an IgE-Binding Protein. *Bioscience, Biotechnology, and Biochemistry* 75:556–560. doi: 10.1271/bbb.100778
- Haydak MH (1970) Honey Bee Nutrition. *Annual Review of Entomology* 15:143–156. doi: 10.1146/annurev.en.15.010170.001043
- Honda Y, Fujita Y, Maruyama H, et al. (2011) Lifespan-extending effects of royal jelly and its related substances on the nematode *Caenorhabditis elegans*. *PloS one* 6:e23527. doi: 10.1371/journal.pone.0023527
- Inoue S (2003) Royal Jelly prolongs the life span of C3H/HeJ mice: correlation with reduced DNA damage. *Experimental Gerontology* 38:965–969. doi: 10.1016/S0531-5565(03)00165-7
- Iwasa H, Yu S, Xue J, Driscoll M (2010) Novel EGF pathway regulators modulate *C. elegans* healthspan and lifespan via EGF receptor, PLC-gamma, and IP3R activation. *Aging cell* 9:490–505. doi: 10.1111/j.1474-9726.2010.00575.x
- Kamakura M (2011) Royalactin induces queen differentiation in honeybees. *Nature* 473:478–483. doi: 10.1038/nature10093

- Kamakura M (2002) Signal transduction mechanism leading to enhanced proliferation of primary cultured adult rat hepatocytes treated with royal jelly 57-kDa protein. *Journal of biochemistry* 132:911–919. doi: 10.1093/oxfordjournals.jbchem.a003304
- Kenyon CJ (2010) The genetics of ageing. *Nature* 464:504–512. doi: 10.1038/nature08980
- Kimura Y, Washino N, Yonekura M (1995) N-linked sugar chains of 350-kDa royal jelly glycoprotein. *Bioscience, biotechnology, and biochemistry* 59:507–509. doi: 10.1271/bbb.59.507
- Kirkland JL (2013) Translating advances from the basic biology of aging into clinical application. *Experimental Gerontology* 48:1–5. doi: 10.1016/j.exger.2012.11.014
- Klass MR (1983) A method for the isolation of longevity mutants in the nematode *Caenorhabditis elegans* and initial results. *Mechanisms of ageing and development* 22:279–286.
- Lattorff HMG, Moritz RFA (2013) Genetic underpinnings of division of labor in the honeybee (*Apis mellifera*). *Trends in Genetics* 29:641–648. doi: 10.1016/j.tig.2013.08.002
- Lemmon M, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. *Cell* 141:1117–1134. doi: 10.1016/j.cell.2010.06.011
- Liu G, Rogers J, Murphy CT, Rongo C (2011) EGF signalling activates the ubiquitin proteasome system to modulate *C. elegans* lifespan. *The EMBO journal* 30:2990–3003. doi: 10.1038/emboj.2011.195
- López-Otín C, Blasco M, Partridge L, et al. (2013) The hallmarks of aging. *Cell* 153:1194–1217. doi: 10.1016/j.cell.2013.05.039
- Lucanic M, Lithgow GJ, Alavez S (2013) Pharmacological lifespan extension of invertebrates. *Ageing research reviews* 12:445–458. doi: 10.1016/j.arr.2012.06.006
- De Magalhães JP, Wuttke D, Wood SH, et al. (2012) Genome-environment interactions that modulate aging: powerful targets for drug discovery. *Pharmacological reviews* 64:88–101. doi: 10.1124/pr.110.004499
- Majtán J, Kováčová E, Bíliková K, Simúth J (2006) The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on TNF α release. *International immunopharmacology* 6:269–278. doi: 10.1016/j.intimp.2005.08.014
- Meelkop E, Temmerman L, Janssen T, et al. (2012) PDF receptor signaling in *Caenorhabditis elegans* modulates locomotion and egg-laying. *Molecular and cellular endocrinology* 361:232–240. doi: 10.1016/j.mce.2012.05.001
- Niu K, Guo H, Guo Y, et al. (2013) Royal jelly prevents the progression of sarcopenia in aged mice in vivo and in vitro. *The journals of gerontology Series A, Biological sciences and medical sciences* 68:1482–1492. doi: 10.1093/gerona/glt041
- Page RE, Peng CY (2001) Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Experimental Gerontology* 36:695–711.

- Qu W, Ren C, Li Y, et al. (2011) Reliability analysis of the Ahringer *Caenorhabditis elegans* RNAi feeding library: a guide for genome-wide screens. *BMC genomics* 12:2–8. doi: 10.1186/1471-2164-12-170
- R Development Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>.
- Rajalingam K, Dikic I (2011) Healthy ageing through regulated proteostasis. *The EMBO journal* 30:2983–2985. doi: 10.1038/emboj.2011.237
- Rongo C (2011) Epidermal growth factor and aging: a signaling molecule reveals a new eye opening function. *Aging* 3:896–905.
- Rual J-F, Ceron J, Koreth J, et al. (2004) Toward Improving *Caenorhabditis elegans* Phenome Mapping With an ORFeome-Based RNAi Library. *Genome Research* 14:2162–2168. doi: 10.1101/gr.2505604
- Schmitzová J, Klaudivy J, Albert S, et al. (1998) A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cellular and molecular life sciences : CMLS* 54:1020–1030. doi: 10.1007/s000180050229
- Simonetta SH, Golombek D a (2007) An automated tracking system for *Caenorhabditis elegans* locomotor behavior and circadian studies application. *Journal of neuroscience methods* 161:273–280. doi: 10.1016/j.jneumeth.2006.11.015
- Šimúth J (2001) Some properties of the main protein of honeybee (*Apis mellifera*) royal jelly. *Apidologie* 32:69–80. doi: 10.1051/apido:2001112
- Solis GM, Petrascheck M (2011) Measuring *Caenorhabditis elegans* life span in 96 well microtiter plates. *Journal of visualized experiments : JoVE*. doi: 10.3791/2496
- Spannhoff A, Kim YK, Raynal NJ-M, et al. (2011) Histone deacetylase inhibitor activity in royal jelly might facilitate caste switching in bees. *EMBO reports* 12:238–243. doi: 10.1038/emboj.2011.9
- Takenaka T (1982) Chemical composition of royal jelly. *Honeybee Science* 3:69–74.
- Wood WB (1988) *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, New York
- Yu S, Driscoll M (2011) EGF signaling comes of age: promotion of healthy aging in *C. elegans*. *Experimental Gerontology* 46:129–134. doi: 10.1016/j.exger.2010.10.010

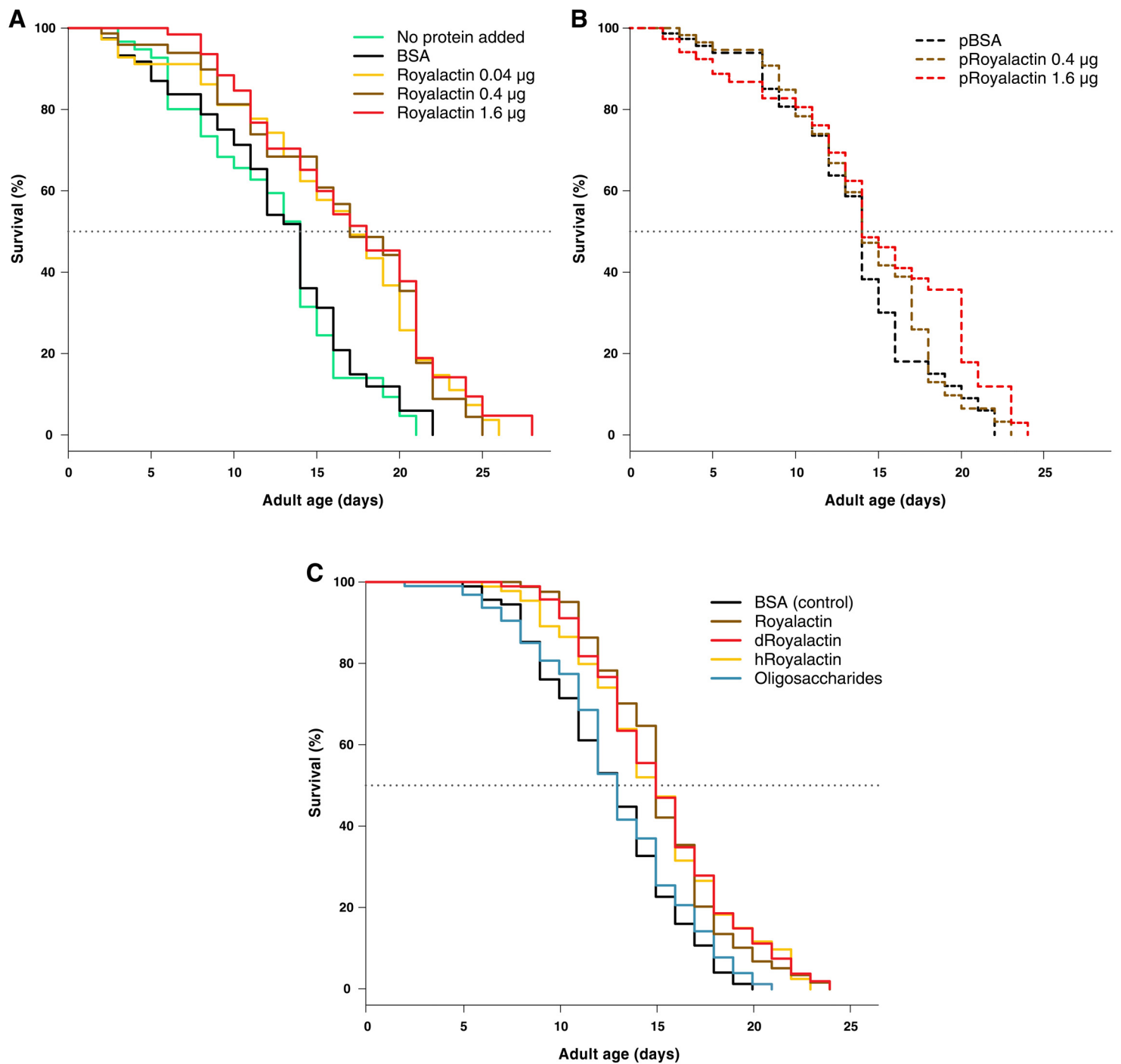


Fig. 1. Royalactin and deglycosylated royalactin (dRoyalactin), but not BSA nor thermolysin-treated royalactin (pRoyalactin), prolong lifespan of *C. elegans*. **(A)** 5 μg BSA mL^{-1} has no significant effect on *C. elegans* lifespan ($p_{\log\text{-rank}} = 0.668$; $p_{\text{cox}} = 0.675$), compared to the condition with no additional protein. All three tested concentrations of royalactin significantly extend lifespan of *C. elegans*, compared to both a control in which no protein was added ($p_{\text{cox}(0.04 \mu\text{g})} = 4.15\text{E-}4$; $p_{\text{cox}(0.4 \mu\text{g})} = 9.19\text{E-}4$; $p_{\text{cox}(1.6 \mu\text{g})} = 5.75\text{E-}5$) and a control supplemented with BSA (Table 1). **(B)** After thermolysin-treatment, the strong lifespan-extending effect of royalactin is abolished. There is no significant difference in survival between both pRoyalactin conditions and the pBSA condition ($p_{\log\text{-rank}(\text{pRoyalactin } 0.4 \mu\text{g})} = 0.409$; $p_{\log\text{-rank}(\text{pRoyalactin } 1.6 \mu\text{g})} = 0.101$). **(C)** Royalactin retains its lifespan-extending effect after deglycosylation ($p_{\log\text{-rank}} = 2.02\text{E-}5$) and mild heat-treatment ($p_{\log\text{-rank}} = 1.25\text{E-}4$), whereas the isolated oligosaccharides do not extend lifespan ($p_{\log\text{-rank}} = 0.415$).

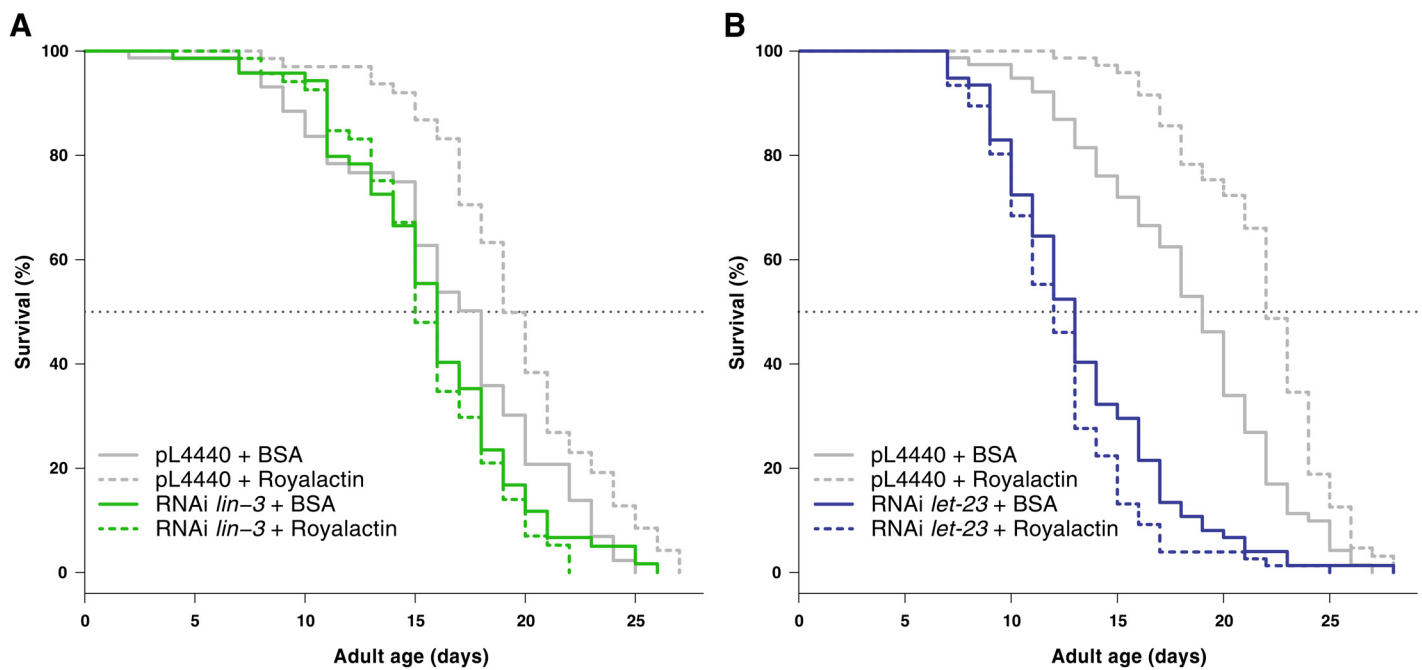


Fig. 2. Knockdown of the EGF homolog *lin-3* (**A**) or its receptor *let-23* (**B**) abolishes the lifespan-extending effect of royalactin on *C. elegans* ($p_{\text{cox}} = 3.17\text{E-}3$ and $2.30\text{E-}5$, respectively).

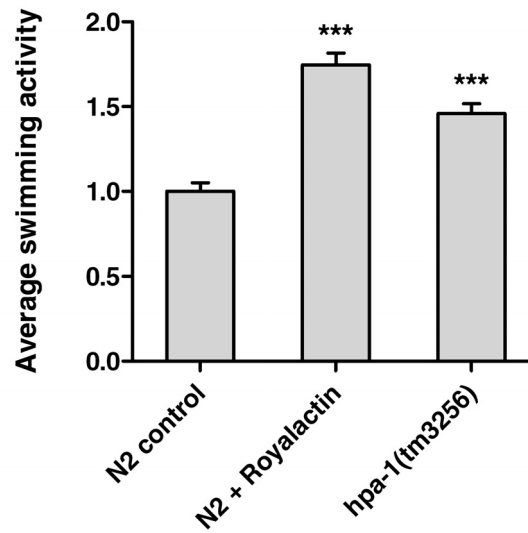


Fig. 3. Royalactin enhances the swimming rate in wild-type *C. elegans* during early to mid-adulthood ($p = 5.41\text{E-}9$). Means were normalized relative to the wild-type control. As a positive control we used the *hpa-1(tm3256)* deletion mutant (*high performance in advanced age*). Error bars indicate SEM, *** $p < 0.001$. Each group consists of 14 replicates with 30 animals in each replicate.

Table 1. Royalactin extends mean and median lifespan of *C. elegans*, independent of its N-linked oligosaccharides.

| Condition | N | Mean lifespan (days \pm SEM) | Increase versus control ^a | p_{cox} | Median lifespan (days) |
|--------------------------------|----|--------------------------------|--------------------------------------|------------------|------------------------|
| A. Intact royalactin | | | | | |
| Royalactin 0.04 μg | 72 | 16.1 \pm 0.84 | 26.8% *** | 6.82E-4 | 17 |
| Royalactin 0.4 μg | 76 | 16.4 \pm 0.90 | 29.1% ** | 1.62E-3 | 17 |
| Royalactin 1.6 μg | 74 | 17.0 \pm 0.80 | 33.9% *** | 8.87E-5 | 18 |
| BSA | 78 | 12.7 \pm 0.68 | - | - | 14 |
| No protein added | 73 | 12.4 \pm 0.74 | -2.4% N.S. | 0.675 | 14 |
| B. Protease-treated royalactin | | | | | |
| pBSA | 76 | 13.6 \pm 0.65 | - | - | 14 |
| pRoyalactin 0.4 μg | 73 | 14.3 \pm 0.66 | 5.1% N.S. | 0.494 | 14 |
| pRoyalactin 1.6 μg | 75 | 14.9 \pm 0.83 | 9.6% N.S. | 0.063 | 14 |
| C. Deglycosylated royalactin | | | | | |
| dRoyalactin | 96 | 15.3 \pm 0.43 | 20.5% *** | 1.84E-5 | 15 |
| hRoyalactin | 95 | 15.0 \pm 0.47 | 18.1% *** | 9.17E-5 | 15 |
| Royalactin | 97 | 15.3 \pm 0.38 | 20.5% *** | 6.69E-5 | 15 |
| Oligosaccharide | 99 | 12.9 \pm 0.41 | 1.6% N.S. | 0.427 | 13 |
| BSA (control) | 98 | 12.7 \pm 0.38 | - | - | 13 |

^a For all conditions with intact royalactin, dRoyalactin, hRoyalactin or oligosaccharides supplemented to the medium, the condition BSA was used as control. For all conditions with pRoyalactin in the NGM plates, pBSA was used as control. N the total amount of tested worms, SEM the standard error of the mean, N.S. not significant, ** $p_{\text{cox}} < 0.01$, *** $p_{\text{cox}} < 0.001$.

Table 2. Lifespan extension by royalactin requires LIN-3 and LET-23.

| Condition | N | Mean lifespan (days \pm SEM) | Increase versus control ^a | Median lifespan (days) |
|-----------------------------------|----|-----------------------------------|---|---------------------------|
| A. <i>lin-3</i> (EGF) knockdown | | | | |
| pL4440 + Royalactin | 73 | 19.5 \pm 0.50 | 17.5% ** | 19 |
| pL4440 + BSA | 77 | 16.6 \pm 0.64 | - | 17 |
| RNAi <i>lin-3</i> + Royalactin | 74 | 15.5 \pm 0.43 | -1.9% ^{N.S.} | 15 |
| RNAi <i>lin-3</i> + BSA | 74 | 15.8 \pm 0.52 | - | 16 |
| B. <i>let-23</i> (EGFR) knockdown | | | | |
| pL4440 + Royalactin | 85 | 21.8 \pm 0.41 | 19.1% *** | 22 |
| pL4440 + BSA | 86 | 18.3 \pm 0.53 | - | 19 |
| RNAi <i>let-23</i> + Royalactin | 86 | 12.3 \pm 0.38 | -8.2% ^{N.S.} | 12 |
| RNAi <i>let-23</i> + BSA | 80 | 13.4 \pm 0.47 | - | 13 |

^a For 'pL4440' we used the condition 'pL4440 + BSA' as control, for 'RNAi *lin-3*' we used 'RNAi *lin-3* + BSA' as control and for 'RNAi *let-23*' we used 'RNAi *let-23* + BSA' as control. Hence, the 'increase versus control' can be considered as the increase in lifespan as a result of royalactin supplementation, in the absence or presence of gene knockdown of *lin-3* (A) or *let-23* (B). N the total amount of tested worms, SEM Standard error of the mean, ** $p_{\text{cox}} < 0.01$, *** $p_{\text{cox}} < 0.001$, ^{N.S.} not significant, *p*-values are shown in appendix B.6.

Appendix A.

Extraction of royalactin and quality-control

Abbreviations

| | |
|-----------|--|
| ACN | Acetonitrile |
| DTT | Dithiothreitol |
| IAA | Idoacetamide |
| TFA | Trifluoroacetic acid |
| MALDI-TOF | Matrix-assisted laser desorption and ionization time-of-flight |
| MQ | Milli-Q water |
| MS | Mass spectrometry |
| PMF | Peptide mass fingerprinting |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |

A.1 Ultracentrifugation of RJ

Royal jelly was separated into three distinct fractions by centrifuging during 5 hours at 245,000 g and 6°C (Optima LE-80K Ultracentrifuge, SW 65 Ti rotor, Beckman Coulter, Nyon, Switzerland). The middle fraction, composed of a yellow viscous fluid, was resuspended in two volumes of Milli-Q water (MQ, Milli-Q Advantage A10 water purification system, 0.22 µm filter, Millipore, Molsheim, France). After 1 h of stirring at 20°C, the milky-white suspension was separated by centrifuging for 30 min at 28,880 g and 4°C (Sorvall RC 6+ centrifuge, F13S-14X50CY rotor, Thermo Scientific, Waltham, USA). This resulted in two fractions: on one hand a sediment of protein aggregates, on the other an opalescent micro-emulsion (with a slight yellow tint). By centrifuging the micro-emulsion (245,000 g, 5h, 6°C) we obtained a clear supernatant and a stiff golden-yellow gel (RJ-gel) on the bottom of the centrifuge tube. After removing the supernatant, the RJ-gel was freeze-dried using a Speedvac system (Savant SVC100H, Savant Instruments Inc, Farmingdale, USA). This process to obtain a RJ-gel – which is rich in royalactin (MRJP1) – corresponds largely to the one described by Simuth (2001).

A small amount of RJ-gel (20 μ g) was dissolved in MQ and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify the presence of royalactin. The RJ-gel indeed contains a relative large amount of 55-57 kDa protein (data not shown). We also found a distinct protein band at \sim 5 kDa, indicating the presence of apisimin, which forms a stable complex with MRJP1 in RJ (Tamura et al. 2009). While the most intense band was the one corresponding to royalactin, further purification was needed due to the presence of additional (albeit weak) protein bands (presumably caused by a minor amount of MRJP3, MRJP5 and to a lesser extend also MRJP4 and MRJP2).

A.2 Reversed-phase high-performance liquid chromatography (RP-HPLC) of RJ-gel

Royalactin was further purified by RP-HPLC of the gel sediment (RJ-gel). We first performed an ‘analytical run’, using a small amount of RJ-gel, in which the different peaks were captured in four separate fractions for subsequent identification through mass spectrometry (MS). Three mg of RJ-gel was dissolved in 2% acetonitrile (ACN, Fluka) and 0.1% trifluoroacetic acid (TFA, Sigma) and brought onto a C4 Symmetry™ 300 Column (4.6 mm x 250 mm, 5 μ m, 300 Å, Waters, Milford, USA) while running a linear gradient of 2% to 80% ACN in 35 minutes. The four different RP-HPLC fractions were freeze-dried and prepared for identification through peptide mass fingerprinting (PMF; see A.3-A.5).

After confirming the presence of royalactin in fraction 3 and 4, we used a Delta-Pack C4 Prep Column (25 x 10 mm, 15 μ m, 100 Å, Waters) with a linear ACN gradient from 2% to 80% in 30 min to obtain larger amounts of royalactin (for usage in the lifespan assays) in a similar way. The RP-HPLC fractions from this ‘preparative run’ were also subjected to PMF analysis. Protein concentrations were determined using a Qubit protein assay kit (Qubit 1.0 Fluorometer, Invitrogen, Paisley, Scotland).

A.3 Tryptic digest of RP-HPLC fractions

RP-HPLC fractions were subjected to hydrolysis by trypsin for subsequent identification through MS. For this, a volume of each RP-HPLC fraction containing ca. 190 μ g of protein was added to LoBind tubes (Eppendorf, Hamburg, Germany). In case the protein concentration was too low to detect (i.e., fraction 1 of the analytical run; $< 20 \mu\text{g protein mL}^{-1}$) the total volume was added.

Samples were freeze-dried and incubated in 50 μ L 30% ACN with 6.6 mM dithiothreitol (DTT, GE Healthcare Life Sciences, Uppsala, Sweden) for 5 minutes at room temperature.

After lyophilising the solution, samples were dissolved in 50 μ L 30% ACN with 55 mM idoacetamide (IAA, Applichem GmbH, Darmstadt, Germany) and incubated for 7 minutes, in the dark, before drying the samples. Next, the samples were dissolved in 150 μ L trypsin digestion buffer (50 mM NH_4HCO_3 , 50 mM CaCl_2) with 1.27 μ g sequencing grade modified trypsin (Promega, Madison, USA), corresponding to an enzyme to substrate ratio of 1:150. Samples were incubated at 37°C during 10 h while vigorously shaking, after which they were again freeze-dried.

A.4 Sample preparation for MALDI-TOF

Tryptic samples were subsequently concentrated and desalted using C18 ZipTip pipette tips (Millipore). Tryptic peptides were eluted in 5 μ L of 70% ACN, 0.1% formic acid (Sigma). For each sample, 1 μ L of tryptic peptides and 1 μ L of matrix (α -cyano-4-hydroxycinnamic acid dissolved in 60% ACN and 0.5% TFA) were spotted on a ground steel MALDI plate (Bruker Daltonics) and allowed to air dry.

A.5 Identification of proteins through *peptide mass fingerprinting* (PMF)

Mass spectrometry was performed on an Ultraflex II matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics) operated in reflectron positive ion mode. The instrument was calibrated using *peptide calibration standard II* (Bruker Daltonics). Mass spectra were generated in the software FlexControl (version 2.4, Bruker Daltonics) while FlexAnalysis (version 3.0, Bruker Daltonics) was used to generate a *peak list* (with a monoisotopic mass and quality factor for each peak). Proteins were identified through PMF using BioTools (version 3.1, Bruker Daltonics) linked to Mascot (version 1.0, Matrix Science). A mass tolerance of 200 ppm was used in all searches and we allowed one missed cleavage per peptide. Carbamidomethylation (C) and oxidation (M) were selected as fixed and variable modifications respectively. We searched the manually annotated and reviewed Swiss-Prot database for potential protein identifications. Hits were considered significant at a Mascot protein score greater than 70. This method allowed for detection of royalactin down to amounts of ca. 150 fmol.

In fraction 3 and 4 of the analytical RP-HPLC run - and fraction 7, 8 and 9 of the preparative RP-HPLC run - we identified royalactin (MRJP1) through PMF MS (see Table A.1). The presence of royalactin was confirmed once more (E-value 0.0076; Score 78; 21 matches; 33%

sequence coverage) through PMF with thermolysin as protease instead of trypsin (cf. thermolysin-treatment described in 2.2).

Table A.1 Identification of royalactin in the RP-HPLC fractions through PMF MS. The ‘E-value’ is an estimate for the amount of matches you would find, with a score greater than the found value, based on chance alone. The Mascot protein ‘score’ is a logarithmic conversion of the E-value. The higher the protein score, the lower the probability that the identification is purely chance-based. ‘Matches’ indicates the amount of observed peptides fragments (corresponding to the identified protein), while ‘sequence coverage’ corresponds to the amount of observed amino acid residues divided by the total length of the protein.

| Sample | Identification | E-value | Score | Matches | Sequence coverage (%) |
|------------------------|----------------|---------|-------|---------|-----------------------|
| Analytical fraction 3 | MRJP1_APIME | 2.7E-5 | 103 | 24 | 60 |
| Analytical fraction 4 | MRJP1_APIME | 1.1E-3 | 87 | 23 | 60 |
| Preparative fraction 7 | MRJP1_APIME | 1.1E-7 | 89 | 13 | 28 |
| Preparative fraction 8 | MRJP1_APIME | 2.9E-12 | 135 | 13 | 26 |
| Preparative fraction 9 | MRJP1_APIME | 1.8E-14 | 157 | 14 | 29 |

A.6 SDS-PAGE of the extracted royalactin

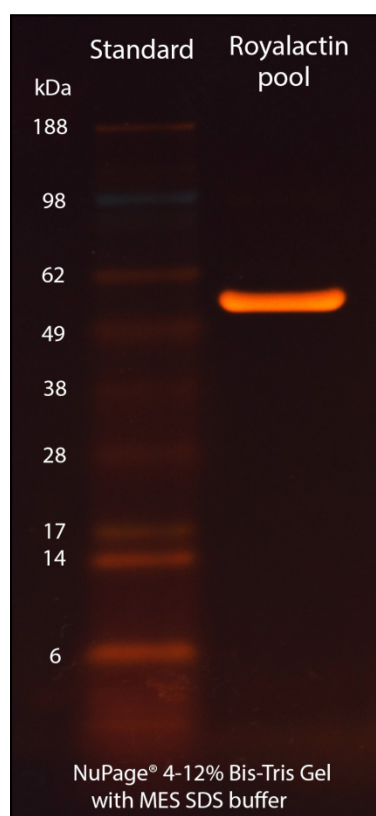


Fig. A.1 SDS-PAGE of the purified royalactin. Left lane: SeeBlue Plus2 Pre-stained Standard (with sizes in kDa). Right lane: 9 µg of the pooled royalactin (which was used in the lifespan assays).

After pooling royalactin for usage in the lifespan experiments, we performed SDS-PAGE to assess the quality of our extraction. We mixed 9 µg of the pooled royalactin with NuPAGE LDS sample buffer (containing Coomassie G-250 and Phenol Red as tracking dyes) and NuPAGE reducing agent (with a final concentration of 50 mM DTT). After heating the sample at 70°C during 10 min, we applied it onto a NuPAGE 4-12% Bis-Tris Midi polyacrylamide gel (Life Technologies, 12+2 wells, 1.0 mm thick). We also applied 10 µL of the SeeBlue Plus2 Pre-stained Standard (Life Technologies) in a neighboring lane, to allow easy visualization of protein molecular weight. Next we subjected the gel to 45 min at 200V (constant, 200 mA) using the Xcell4 SureLock Midi-Cell system with 1x NuPAGE MES SDS running buffer (Life Technologies). After rinsing the gel with MQ, we colored the proteins by incubating the gel for one hour in ca. 20 mL SimplyBlue™ Safestain (Invitrogen) while gently rocking. After rinsing the gel three times with MQ during 5 minutes, we

took a picture with a Nikon D5100. To obtain a clearer image of the protein bands, we digitally inverted the colors of the picture.

This resulted in a single protein band (Fig. A.1) where one would expect royalactin (55-57 kDa). Other *major royal jelly proteins* (MRJPs), that were present in the RJ-gel prior to RP-HPLC (see A.1), show no visible bands. This further corroborates a successful extraction of royalactin from RJ, as already suggested by our MS data (see A.5).

A.7 Blue native PAGE

To assess whether our purified MRJP1 is present as a monomer or oligomer, we subjected it to non-reducing and non-denaturing gel electrophoresis, very similar to Tamura et al. (2009).

We used the NativePAGE Novex Bis-Tris Gel system (Life Technologies) to estimate the molecular mass of native MRJP1. Fifteen μ g of purified MRJP1 (see A.1 – A.2) was mixed with NativePAGE sample buffer (Life Technologies) and Coomassie Brilliant Blue G-250 (Sigma) according to the manufacturer's instructions and brought onto a NativePAGE Novex 4-16% Bis-Tris Protein Gel (1.0 mm, 10 wells, Life Technologies). The gel was subjected to 140 minutes at 150V using the XCell™ SureLock™ Mini-Cell system (Life Technologies). As a molecular weight marker we used the NativeMark Unstained Protein Standard (Life Technologies). After fixating the gel in a solution with 50% (v/v) methanol and 10% (v/v) acetic acid for one hour, the gel was stained with SimplyBlue Safestain (Life Technologies) as described previously (see A.6). Note that weight estimation via this electrophoretic method is not as accurate as with SDS-PAGE, since electrophoretic mobility also depends on protein shape and not exclusively on protein size. More information concerning the used method, also known as Blue native PAGE, can be found in the literature (Wittig et al. 2006).

Our purified MRJP1 shows a strong band (Fig. A.2, between 66 kDa and 146 kDa), corresponding to where one would expect monomeric MRJP1 (Tamura et al. 2009). A band around 290 kDa, which is characteristic for MRJP1 in the oligomeric form (Tamura et al. 2009), was not observed. In summary, this

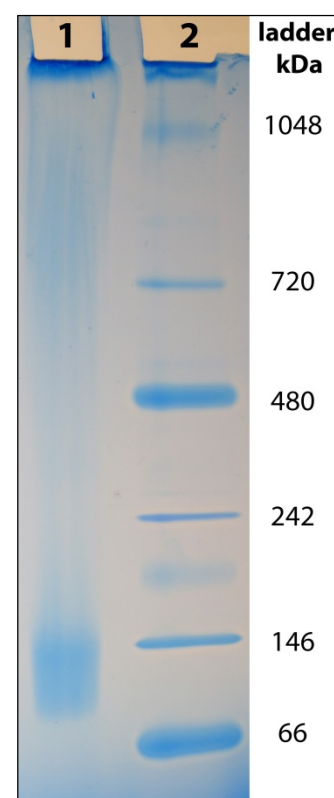


Fig. A.2 Native PAGE of purified royalactin. Lane one: fifteen μ g of purified royalactin (which was used in the lifespan assays). Lane two: NativeMark Unstained Protein Standard (with sizes in kDa).

result indicates that the MRJP1 obtained through our purification method is predominantly present in the monomeric form (which is referred to as royalactin).

A.8 Verification of deglycosylation

We performed N-linked deglycosylation of purified royalactin (MRJP1 monomer) under non-denaturing conditions (see 2.3) and analyzed the resulting product by gel electrophoresis.

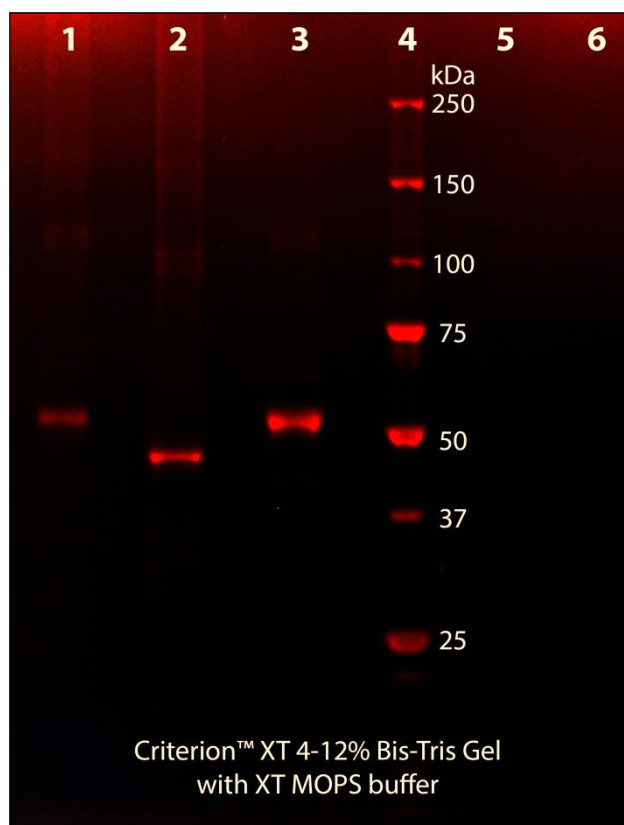


Fig. A.3 SDS-PAGE to verify deglycosylation.

Lane 1: 0.5 μ g hRoyalactin, lane 2: 0.5 μ g dRoyalactin, lane 3: 0.5 μ g royalactin, lane 4: 10 μ L Precision Plus Protein Unstained Standard (with sizes in kDa), lane 5: 0.6 μ L filtrate from hRoyalactin (equivalent to 0.5 μ g protein), lane 6: 0.6 μ L filtrate from dRoyalactin (equivalent to 0.5 μ g protein).

SDS-PAGE was performed using a Criterion™ XT Bis-Tris gel (4–12%, 1.0 mm, 26 wells, Bio-Rad) with XT MOPS running buffer (Bio-Rad) in accordance with the manufacturer's instructions. As a molecular weight ladder we used the Precision Plus Protein™ Unstained Standard (Bio-Rad). The gel was stained and imaged as described earlier (A.6).

The removal of carbohydrate moieties from MRJP1 has been reported in literature (albeit under denaturing conditions: Ohashi et al. 1997; Kamakura et al. 2001; Kamakura 2002) and results in a characteristic shift in apparent molecular

weight from ~56 kDa to ~47 kDa (SDS-PAGE). We observed an analogous shift (Fig. A.3: lane 2 versus lane 3), indicating successful deglycosylation. The oligosaccharide fractions obtained via filtering of the heat-treated and

deglycosylation reaction mixtures (see 2.3), show no clear bands (Fig. A.3: lane 5 and lane 6).

References

- Kamakura M (2002) Signal transduction mechanism leading to enhanced proliferation of primary cultured adult rat hepatocytes treated with royal jelly 57-kDa protein. *Journal of biochemistry* 132:911–919. doi: 10.1093/oxfordjournals.jbchem.a003304
- Kamakura M, Fukuda T, Fukushima M, Yonekura M (2001) Storage-dependent degradation of 57-kDa protein in royal jelly: a possible marker for freshness. *Bioscience, biotechnology, and biochemistry* 65:277–284. doi: 10.1271/bbb.65.277
- Ohashi K, Natori S, Kubo T (1997) Change in the mode of gene expression of the hypopharyngeal gland cells with an age-dependent role change of the worker honeybee *Apis mellifera* L. *European journal of biochemistry / FEBS* 249:797–802. doi: 10.1111/j.1432-1033.1997.t01-1-00797.x
- Šimúth J (2001) Some properties of the main protein of honeybee (*Apis mellifera*) royal jelly. *Apidologie* 32:69–80. doi: 10.1051/apido:2001112
- Tamura S, Amano S, Kono T, et al. (2009) Molecular characteristics and physiological functions of major royal jelly protein 1 oligomer. *Proteomics* 9:5534–5543. doi: 10.1002/pmic.200900541
- Wittig I, Braun H-P, Schägger H (2006) Blue native PAGE. *Nature protocols* 1:418–428. doi: 10.1038/nprot.2006.62

Appendix B.

Additional information regarding the lifespan experiments

Abbreviations

| | |
|--------|--|
| FUdR | 5-fluoro-2'-deoxyuridine |
| IPTG | Isopropyl β -D-1-thiogalactopyranoside |
| LB | Lysogeny broth |
| LB Amp | Lysogeny broth containing 50 μ g/mL ampicillin |
| MCS | Multiple cloning site |
| MLS | Mean lifespan |

B.1 Composition of *C. elegans* media

The composition of the NGM agar differed between the lifespan experiments. This was partly due to application of RNAi (by feeding) in the second and third experiment, which requires isopropyl β -D-1-thiogalactopyranoside (IPTG) and ampicillin.

- NGM₁ agar: 1.7% (w/v) agar, 50 mM NaCl, 0.25% (w/v) Bacto™ Peptone, 10 μ g/mL cholesterol, 2 mM MgSO₄, 2 mM CaCl₂, 20 mM KH₂PO₄, 5 mM K₂HPO₄.
- NGM₂ and NGM₃ agar: 1.7% (w/v) agar, 50 mM NaCl, 0.50% (w/v) Bacto™ Peptone, 5 μ g/mL cholesterol, 1 mM MgSO₄, 1 mM CaCl₂, 20 mM KH₂PO₄, 5 mM K₂HPO₄, 1 mM IPTG, 50 μ g/mL ampicillin.
- NGM₄ agar: identical to NGM₂ and NGM₃ apart from IPTG which was omitted.

Additional protein (i.e., royalactin and BSA) was always added to the liquid NGM agar (at \pm 50°C) immediately prior to pouring the plates. The same goes for IPTG, ampicillin, CaCl₂, MgSO₄, KH₂PO₄, K₂HPO₄ and cholesterol (added as 5 mg ml⁻¹ in ethanol). After pouring the NGM₂ and NGM₃ agar plates, they were stored for 24 hours in the dark at room temperature, prior to seeding with dsRNA-producing bacteria.

Table B.1 Different conditions used in the first *C. elegans* lifespan experiment. pRoyalactin and pBSA refer to thermolysin-treated royalactin and thermolysin-treated BSA, respectively (see 2.2). All plates were seeded with OP50.

| Name condition | [Royalactin] $\mu\text{g mL}^{-1}$ NGM ₁ | [BSA] $\mu\text{g mL}^{-1}$ NGM ₁ | [pRoyalactin] $\mu\text{g mL}^{-1}$ NGM ₁ | [pBSA] $\mu\text{g mL}^{-1}$ NGM ₁ |
|-------------------------------|--|---|---|--|
| Royalactin 0.04 μg | 0.04 | 4.96 | 0 | 0 |
| Royalactin 0.4 μg | 0.4 | 4.6 | 0 | 0 |
| Royalactin 1.6 μg | 1.6 | 3.4 | 0 | 0 |
| BSA | 0 | 5.0 | 0 | 0 |
| No protein | 0 | 0 | 0 | 0 |
| pBSA | 0 | 0 | 0 | 5.0 |
| pRoyalactin 0.4 μg | 0 | 0 | 0.4 | 4.6 |
| pRoyalactin 1.6 μg | 0 | 0 | 1.6 | 3.4 |

Table B.2 Conditions used in the second *C. elegans* lifespan experiment. Plates were seeded with HT115(DE3) containing an empty pL4440 plasmid or pL4440(*lin-3*).

| Name condition | [Royalactin] $\mu\text{g mL}^{-1}$ NGM ₂ | [BSA] $\mu\text{g mL}^{-1}$ NGM ₂ | Target gene (RNAi) | Extra info |
|--------------------------------|---|--|-----------------------|------------------|
| pL4440 + BSA | 0 | 5 | None | Negative control |
| pL4440 + Royalactin | 1.5 | 3.5 | None | Positive control |
| RNAi <i>lin-3</i> + BSA | 0 | 5 | <i>lin-3</i> | EGF knockdown |
| RNAi <i>lin-3</i> + Royalactin | 1.5 | 3.5 | <i>lin-3</i> | EGF knockdown |

Table B.3 Conditions used in the third *C. elegans* lifespan experiment. Plates were seeded with HT115(DE3) containing an empty pL4440 plasmid or pL4440(*let-23*).

| Name condition | [Royalactin] $\mu\text{g mL}^{-1}$ NGM ₃ | [BSA] $\mu\text{g mL}^{-1}$ NGM ₃ | Target gene (RNAi) | Extra info |
|---------------------------------|---|--|-----------------------|------------------|
| pL4440 + BSA | 0 | 3 | None | Negative control |
| pL4440 + Royalactin | 1.6 | 1.4 | None | Positive control |
| RNAi <i>let-23</i> + BSA | 0 | 3 | <i>let-23</i> | EGFR knockdown |
| RNAi <i>let-23</i> + Royalactin | 1.6 | 1.4 | <i>let-23</i> | EGFR knockdown |

Table B.4 Conditions used in the fourth *C. elegans* lifespan experiment. Note that we seeded the plates with the ampicillin-resistant HT115(DE3) containing an empty pL4440 plasmid, without inducing the production of dsRNA. We did this so we could use ampicillin to reduce potential contamination as a result of the processing of dRoyalactin and hRoyalactin under non-sterile conditions (i.e., 60 h at 37°C). dRoyalactin and hRoyalactin refer to deglycosylated and heat-treated royalactin, respectively (see 2.3).

| Name condition | [Royalactin] $\mu\text{g mL}^{-1}$ NGM ₄ | [dRoyalactin] $\mu\text{g mL}^{-1}$ NGM ₄ | [hRoyalactin] $\mu\text{g mL}^{-1}$ NGM ₄ | [BSA] $\mu\text{g mL}^{-1}$ NGM ₄ | Extra info |
|------------------|---|--|--|--|--------------------------------------|
| Royalactin | 1.6 | 0 | 0 | 1.4 | Positive control (intact royalactin) |
| dRoyalactin | 0 | 1.6 | 0 | 1.4 | Deglycosylated royalactin |
| hRoyalactin | 0 | 0 | 1.6 | 1.4 | Control for heat-degradation |
| Oligosaccharides | 0 | 0 | 0 | 3 | Sugar chains removed from royalactin |
| BSA (control) | 0 | 0 | 0 | 3 | Negative control |

B.2 Seeding agar plates

- Experiment 1: We picked a single colony of *E. coli* OP50 into 30 mL lysogeny broth (LB, Sigma) medium and incubated it overnight at 37°C while shaking. Next, the liquid OP50 culture was applied on fresh NGM₁ agar plates and spread out using sterile technique. The surface of medium-sized plates was covered completely with 150 μL of the bacterial solution, while assay plates were only covered for 2/3th with 30 μL of the bacterial solution (in the centre of the plate, to prevent worms from crawling off during the lifespan assays). After overnight incubation at 37°C, and 24 hours at room temperature, the plates were stored at 4°C or used immediately.
- Experiment 2, 3 and 4: Plates were seeded with an overnight culture of the *E. coli* strain HT115(DE3) (containing the appropriate RNAi construct), grown in LB medium with 50 $\mu\text{g/mL}$ ampicillin (LB Amp). We used 60 μL of bacterial culture per assay plate, and 300 μL per medium-sized plate. Plates were incubated at 37°C overnight, followed by 24 hours at room temperature in the dark before storage at 4°C. To prevent internal hatching (see B.4) we added 5-fluoro-2'-deoxyuridine (FUdR) with a final concentration of 100 μM to the assay plates, ca. 30 min before transferring worms onto them.

B.3 Cycle sequencing of the RNAi constructs

Every RNAi construct (i.e., pL4440 plasmid coding for a specific dsRNA) was checked by DNA sequencing. For this plasmid DNA from the different RNAi clones was extracted with a GenElute™ HP Plasmid Miniprep Kit (Sigma). DNA concentrations were measured with a

Nanodrop spectrophotometer (Nanodrop ND-1000, Isogen Life Science, De Meern, the Netherlands). We then performed a cycle sequencing reaction (Primrose and Twyman 2006) using a BigDye[®] Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, USA) and a TProfessional Standard 96 well Thermocycler (Biometra, Westburg, Germany).

Reactions were performed in a volume of 20 μ L with: 1 μ L of a 3.2 μ M primer solution (pL4440-dest-RNAi-REV universal primer 5'-TGGATAACCGTATTACCGCC-3'), 2 μ L 2.5x *Ready Reaction Premix*, 6 μ L 2.5x *BigDye Sequencing Buffer* and ca. 150-200 ng of plasmid DNA in GenElute[™] HP Miniprep elution buffer (10 mM Tris-HCl, pH 8.5). After an initial denaturation step during 2 min at 96°C, samples were subjected to 25 cycles of 30 s at 95°C (denaturation), 15 s at 50°C (primer hybridization) and 2 min 30 s at 60°C (elongation). After a final elongation step of 3 min, the extension products were precipitated via the Ethanol/EDTA/Sodium acetate method, as described in the manual of the BigDye[®] Terminator Cycle Sequencing kit. Actual DNA sequencing, by capillary electrophoresis and detection of the incorporated fluorescent dideoxynucleotides, took place on a Hitachi 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were aligned with the *C. elegans* genome and/or the pL4440 vector sequence through BLASTn (Madden 2003).

As expected, the “empty pL4440 construct” only contains the unmodified *multiple cloning site* (MCS) of the pL4440 vector between both T7 promoters (Output BLAST: identity 100%, E value 9E-111, max score 401). The pL4440(*unc-22*) and pL4440(*lin-3*) constructs also contain the correct insert in the MCS (Output BLAST: identity 98%, E value 8E-175, max score 614 and identity 99%, E value 0.0, max score 1068, respectively).

The RNAi construct for *let-23* from the Ahringer RNAi feeding library (Kamath et al. 2003) turned out to hold an incorrect insert. The MCS indeed contains a genomic fragment of *C. elegans* (cf. Cosmid F49E12, position 28144-28563; identity 97%, max score 699, E value 0.0), yet it's a sequence ca. 1 million base pairs removed from the *let-23* gene. This result was deposited in the CelRNAi database (Qu et al. 2011) together with information from our first pilot experiment (see B.4). This outcome led us to build our own in-house *let-23* RNAi construct (see B.5; Output BLAST: identity 100%, E value 0.0, max score 1142) to knock down *let-23* in the third lifespan experiment.

B.4 Pilot experiments to confirm RNAi induction

Before the second lifespan assay we performed a pilot experiment to see if RNAi was induced successfully at the desired experimental conditions: at 20°C, with worms on the RNAi plates starting from the L1 stage. When performing RNAi against *unc-22* (see 2.5.4) we observed the expected “twitching” phenotype (Benian et al. 1993; Fire et al. 1998) – both in the presence and absence of royalactin – in ca. 99% of the worms after 4 days at 20°C (4 replicas; >300 animals). Worms fed with the empty pL4440 vector, which leads to the production of a dsRNA fragment corresponding to the MCS of the plasmid, showed no unusual phenotype (as expected).

When feeding worms with the *lin-3* RNAi clone (from the *C. elegans* ORF-RNAi library v1.1) approx. 95% of the worms died after the first day of adulthood. Presumably this was due to the inability to develop a vulva opening (a process in which LIN-3 plays an important role; Hill and Sternberg 1992) and the subsequent internal hatching of eggs (i.e., “bag of worms phenotype”). Adding FUdR to the plates (from the late L4 stage; 100 $\mu\text{M mL}^{-1}$ NGM), which eliminates live progeny, solved this problem.

When feeding worms with the *let-23* RNAi clone from the Ahringer RNAi feeding library (Kamath et al. 2003) there was no difference in phenotype compared to control worms (fed with the RNAi clone containing the empty vector). Nor did we see a “bag of worms” or “vulvaless” phenotype as one would expect when successfully knocking down *let-23*. After DNA sequencing analysis, the RNAi clone turned out to be incorrect (see B.3). This led us to build our own in-house *let-23* RNAi construct (see B.5).

Before the final lifespan experiment we performed another pilot experiment in which we fed wild-type worms with HT115(DE3) containing our in-house *let-23* RNAi construct (pL4440(*let-23*)). Median lifespan was reduced from 18 days to 12 days (-33.3%) compared to a control fed with the empty pL4440 vector (on standard NGM with 1 mM IPTG and 50 $\mu\text{g mL}^{-1}$ ampicillin). Mean lifespan was reduced from 17.3 ± 0.46 to 12.7 ± 0.38 (-26.6%; $p_{\text{cox}} = 3.17\text{E-}11$). These results suggest a successful knockdown of *let-23*, with no complete loss-of-function (cf. the latter is seen with the *let-23(n1045)* mutant, which has an even shorter mean lifespan at 20°C (Liu et al. 2011)).

B.5 Establishing the pL4440(*let-23*) RNAi construct

We amplified a 1049 bp fragment of *let-23* from genomic *C. elegans* DNA by PCR and cloned it into the pL4440 RNAi feeding vector. The method we used is comparable to the one described by Kamath and Ahringer (2003), with a few adjustments.

As target sequence in *let-23*, we chose a region very similar to the region delineated by the GenePair ZK1067.1 (WormBase release WS236, Wormbase ID WBGene00002299), with a slight modification to the anneal site of the reverse primer. Near the 5' end of both primers we incorporated a restriction site (GAGCTC) for the *SacI* restriction endonuclease (Thermo Scientific). This enzyme cuts in the MCS of the pL4440 vector (at position 75), but not in our *let-23* target sequence. We also added a three nucleotide spacer (with sequence CTC) at the 5' end of both primers, which is needed for the proper functioning of *SacI*.

Forward primer = 5'-CTCGAGCTCATGCACGATGATGGTTTGAA-3' (plus strand)

Reverse primer = 5'-CTCGAGCTCCGGTTTGACCATCTTCATTT-3' (minus strand)

Target sequence:

```
ATGCACGATGATGGTTTGAAAATTGTCTACTATCTACAGACCTCTCGGAAATCTTCAAACCTTTTGAACTTCACAAGGAAAAATTTGGGTGCACGAGAACAAGTATT
ATATTGTTATCAAATTGCATCTGGAATGCAATATTAGAGAAACAGCGAGTTGTTACAGAGATTTGGCAACAAGAAATGTTCTTGTGAAGAAGTTCAATCAGTGG
AAATCACTGATTTTGGTCTCTCTAAAATTCTGAAACATGACGCCGACTCAATCACTATCAAATCTGAAAAGTTGCAATCAAATGGCTTGCGATCGAAATCTTCTCC
AAACATTGTTACACCCATGCTTCTGATGTTTGGGCATTTGGAGTCACATGTTGGGAGATTATAACATTTGGACAAAGTCCGTATCAAGGAATGTCGACTGATAGTAT
TCATAATTTCTTGAAAGATGGAACCGCTTTTCCAGCCACCAAACCTGCTCTCAAGACTTGTATCAAGAGCTTCTGAGATGTTGGATGGCGGATCCAAAGTCTCGTC
CTGGTTTGAATATTATATGAAAGATTTAAAGAATTCTGTAAGGTTCCCAACTTTTCTGGAATTTCCAACAAGATTTCTGAATCTGATTTGTGAGCTGAAGAA
CGTTTTTCAAACGGAAGAATCCGAGAAATGTTTGTATGGAATATCGATCCACAGATGTACTTTGATCAAGGAAGTTTACCAAGTATGCCAAGTCTCCAACCTCTAT
GGCAACGGTAAGCATTTTAAATTAGTTTTTTTTTTCGAATATCAAATAATATTTTACAGTTCACAATCCACATGGTGATCTGATGAATCGAATGAATCAGTAAA
CTCATCTAGGTACAAAACGGAGCCTTTTGATTGTAAGTTATTCTAATTTCTTTTCAATTTTATAATTATTTTGATTTTATAGTGGGTCAACCGCACAGGAAGA
TAATTCATATCTTATTCAAAAACCAAGAAGTTCAGCAGTCAGCAGTTTGTATACAGCTGTTACAAATGAAGATGGTCAAACCG
```

We amplified the above target sequence from ca. 100 ng genomic DNA using the PfuUltra II Fusion HS DNA Polymerase (Agilent, Santa Clara, USA), following instructions of the supplier. Successful amplification was checked via Tris-acetate-EDTA agarose gel electrophoresis and staining of the DNA with GelRed™ (Biotium, Hayward, USA). After purification of the PCR product (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany), we cloned the *let-23* fragment into the pL4440 vector (Addgene, Cambridge, USA, <http://www.addgene.org/1654/>) using *SacI* and T4 DNA Ligase (Thermo Scientific). Next we transformed One Shot *E. coli* TOP10 Competent Cells using ampicillin selection. Positive

transformants were screened by PCR (using a T7 primer: 5'-TAATACGACTCACTATAGGG-3') to verify the presence of an insert in the MCS of the pL4440 vector. Plasmid DNA was isolated and sequenced (as described in B.3) to confirm the actual identity of the insert (i.e., the desired target sequence from *let-23*). Finally we transformed *E. coli* HT115(DE3) competent cells, similarly to Kamath and Ahringer (2003), with our in-house pL4440(*let-23*) construct. Positive clones were sequenced once more as described in B.3, with the difference that we now used the M13 Forward (-21) primer (from the BigDye[®] Terminator Cycle Sequencing Kit v1.1) to verify the presence of the correct insert.

B.6 Additional lifespan curves and *p*-values

B.6.1 EGF knockdown

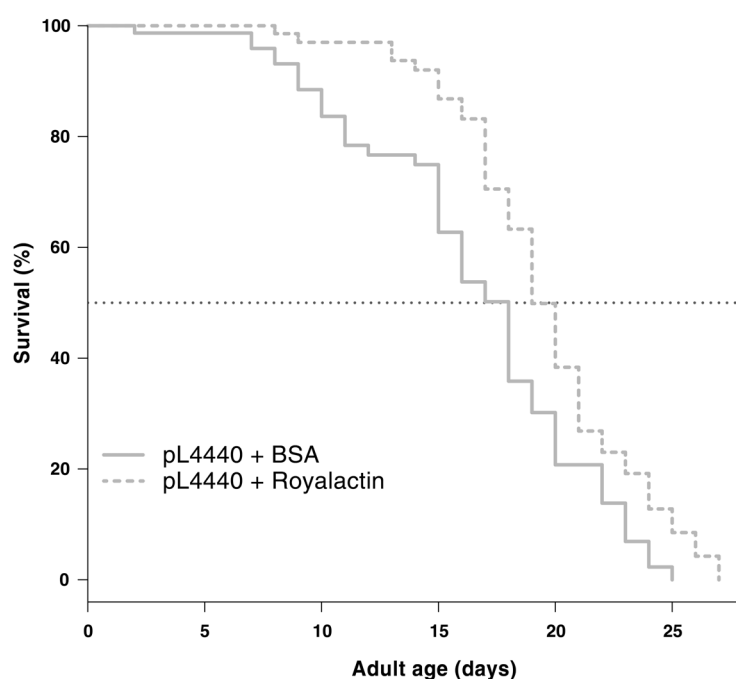


Fig. B.1. The lifespan-extending effect of royalactin is maintained when culturing *C. elegans* in the presence of HT115(DE3) expressing dsRNA corresponding to the MCS of the pL4440 vector during the second lifespan experiment. Worms treated with 1.5 μg royalactin mL^{-1} NGM₂ live significantly longer than those treated with only BSA ($p_{\text{log-rank}} = 0.00254$; $p_{\text{cox}} = 0.00343$).

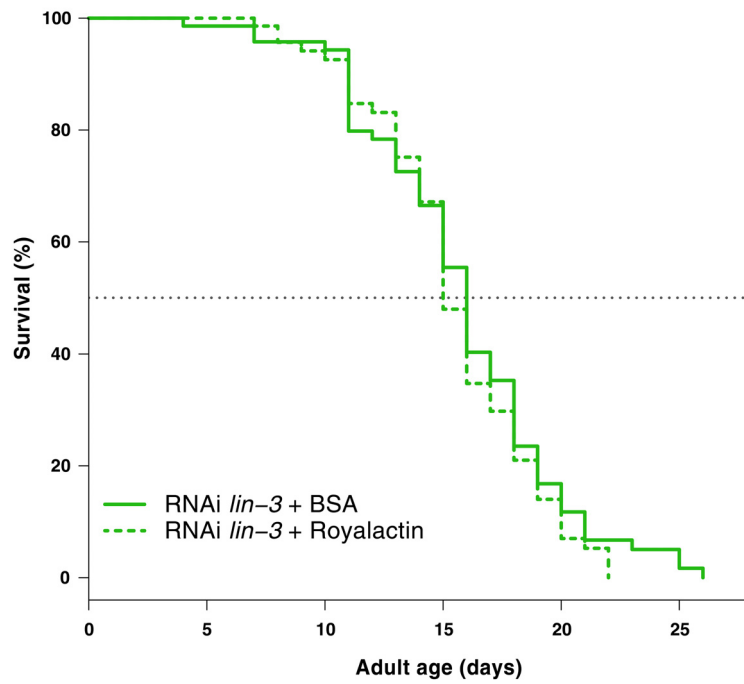


Fig. B.2. When knocking down *lin-3*, which codes for the EGF ligand LIN-3, royalactin-treatment does not result in a significant increase in survival ($p_{\text{log-rank}} = 0.372$; -1.9% MLS).

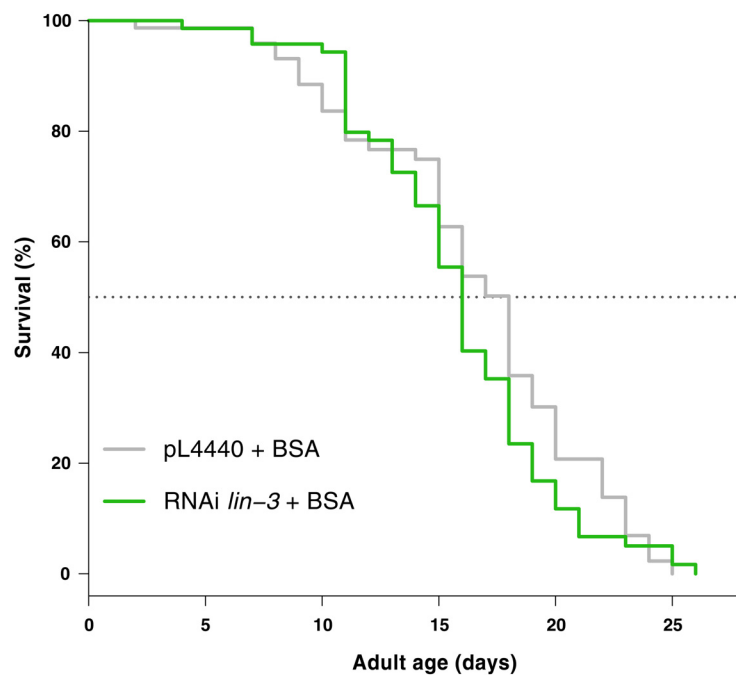


Fig. B.3. Knockdown of *lin-3* causes no significant difference in survival (compared to the control with the empty pL4440 vector) on NGM₂ agar plates supplemented with BSA ($p_{\text{log-rank}} = 0.256$; -4.8% MLS).

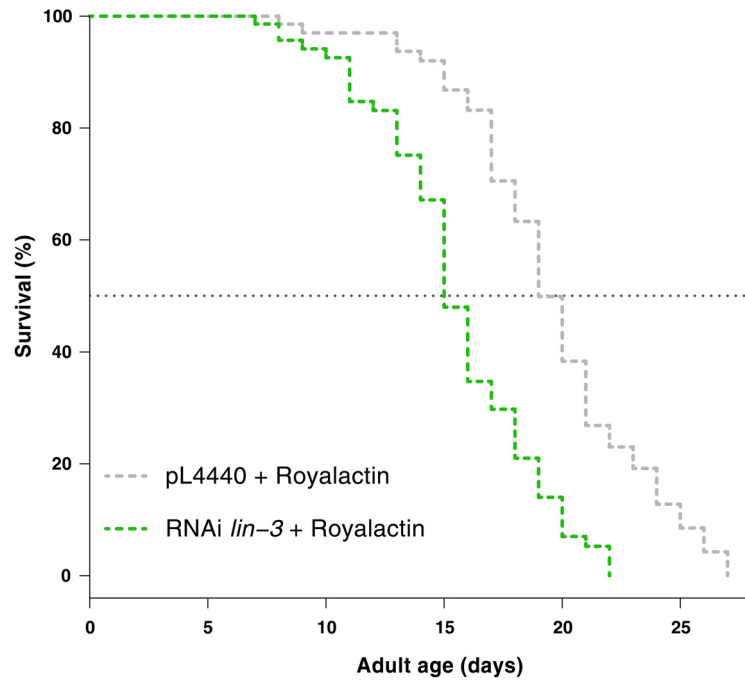


Fig. B.4. On agar plates with $1.5 \mu\text{g royalactin mL}^{-1}$ NGM₂, MLS decreased by 20.5% when knocking down *lin-3* compared to the control fed the empty vector ($p_{\log\text{-rank}} = 1.26\text{E-}8$).

B.6.2. EGFR knockdown

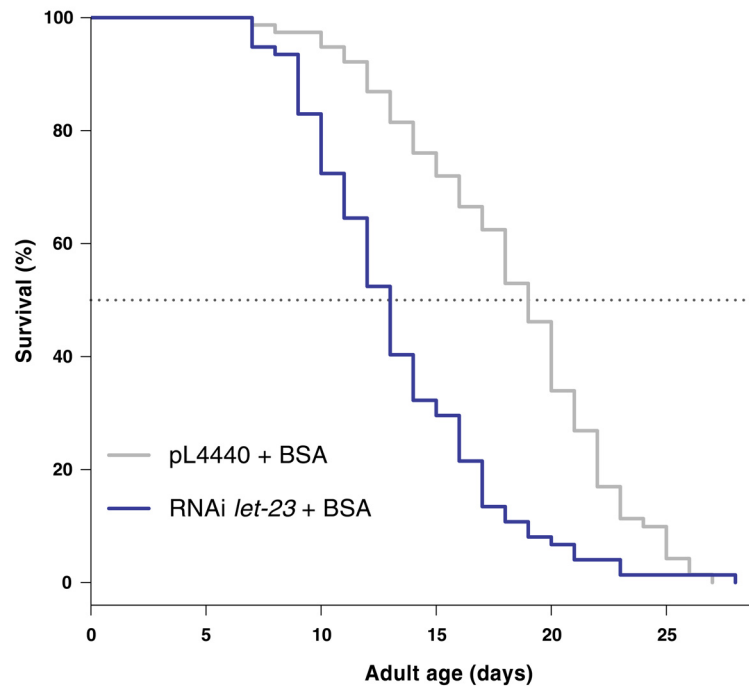


Fig. B.5. Knockdown of *let-23* drastically decreases survival of *C. elegans* on NGM₃ agar plates supplemented with BSA ($p_{\text{cox}} = 1.47\text{E-}8$; $p_{\log\text{-rank}} = 7.29\text{E-}09$; -26.8% MLS).

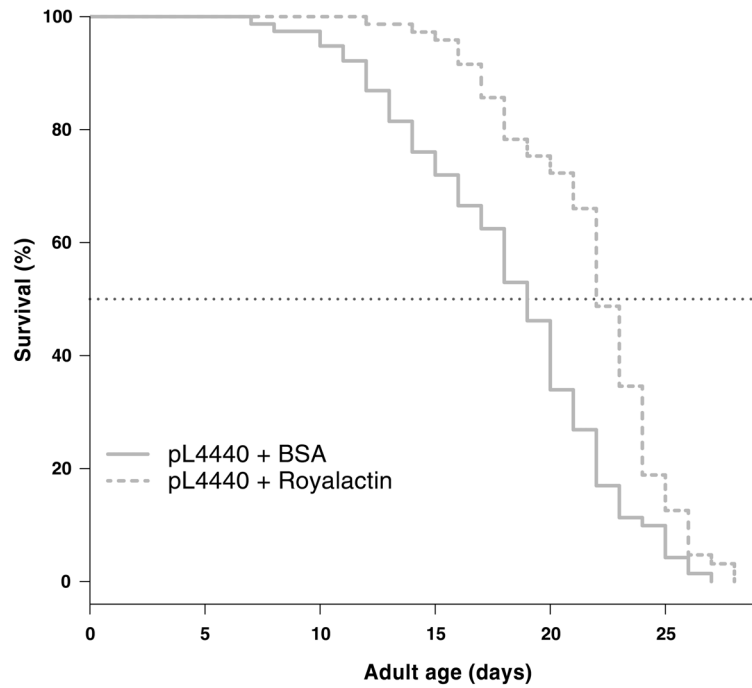


Fig. B.6. The lifespan-extending effect of royalactin is maintained when culturing *C. elegans* in the presence of HT115(DE3) expressing dsRNA corresponding to the MCS of the pL4440 vector during the third lifespan experiment. Worms treated with $1.6 \mu\text{g}$ royalactin mL^{-1} NGM₃ live significantly longer than those treated with only BSA ($p_{\text{cox}} = 5.69\text{E-}4$).

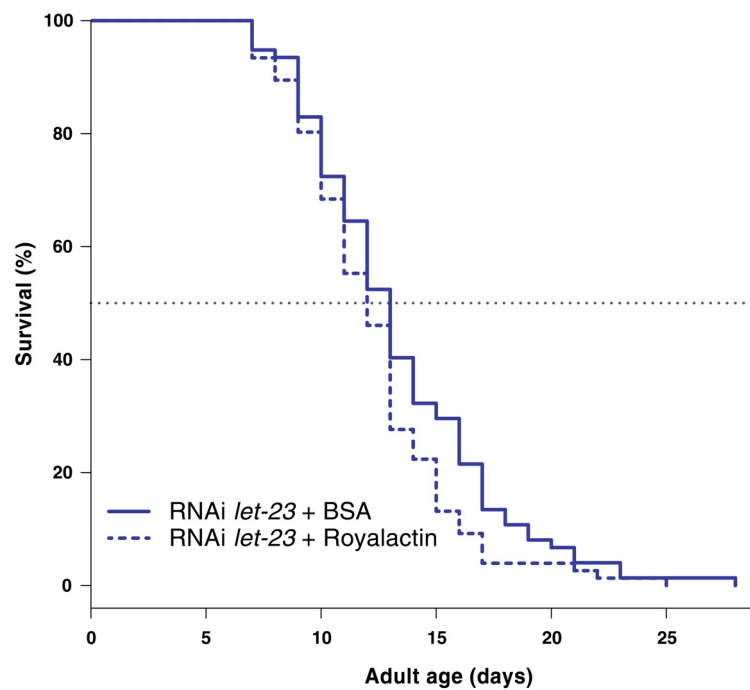


Fig. B.7. When knocking down *let-23* the lifespan-extending effect of royalactin is completely abolished. There is even a slight, albeit non-significant, reduction in MLS of 8.2% ($p_{\text{log-rank}} = 0.062$).

References

- Benian GM, L'Hernault SW, Morris ME (1993) Additional sequence complexity in the muscle gene, *unc-22*, and its encoded protein, twitchin, of *Caenorhabditis elegans*. *Genetics* 134:1097–1104. doi: 10.1074/jbc.M111.271452
- Fire A, Xu S, Montgomery MK, et al. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–811. doi: 10.1038/35888
- Hill RJ, Sternberg PW (1992) The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* 358:470–476. doi: 10.1038/358470a0
- Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* 30:313–321. doi: 10.1016/S1046-2023(03)00050-1
- Kamath RS, Fraser AG, Dong Y, et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421:231–237. doi: 10.1038/nature01278
- Liu G, Rogers J, Murphy CT, Rongo C (2011) EGF signalling activates the ubiquitin proteasome system to modulate *C. elegans* lifespan. *The EMBO journal* 30:2990–3003. doi: 10.1038/emboj.2011.195
- Madden T (2003) The BLAST Sequence Analysis Tool. In: McEntyre, J, Ostell, J (eds) *The NCBI Handbook*. National Center for Biotechnology Information, Bethesda (MD), USA, pp 16.1–16.17
- Primrose SB, Twyman RM (2006) *Principles of Gene Manipulation and Genomics*, 7th ed.
- Qu W, Ren C, Li Y, et al. (2011) Reliability analysis of the Ahringer *Caenorhabditis elegans* RNAi feeding library: a guide for genome-wide screens. *BMC genomics* 12:2–8. doi: 10.1186/1471-2164-12-170